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First genetic survey on the invasive rayed pearl oyster *Pinctada radiata* (Leach, 1814) populations of the Balearic Islands (Western Mediterranean)

Joseba AGUILO-ARCE[1, 2], Joana F. FERRAGUT[1], Lydia PNG-GONZALEZ[3], Aina CARBONELL[3] and María CAPA[1,4]

[1] Department of Biology, Universitat de les Illes Balears, Ctra. de Valldemossa km 7.5, 07122 Palma de Mallorca, Spain
[2] (Present address) Department of Bioscience, Biotechnology and Environment, Università degli Studi di Bari Aldo Moro, Via Orabona 4, 70125 Bari, Italy
[3] Centro Oceanográfico de Baleares (IEO, CSIC), Muelle de Poniente s/n, 07015 Palma de Mallorca, Spain
[4] Balearic Biodiversity Centre, Universitat de les Illes Balears, Ctra. de Valldemossa km 7.5, 07122 Palma de Mallorca, Spain

Corresponding author: María CAPA; maria.capa@uib.es

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Abstract

A survey of the invasive pearl oyster *Pinctada radiata* along the Balearic Archipelago was conducted based on the COI mitochondrial marker. Results confirmed the identity of the specimens as *P. radiata*. The Balearic populations are certainly of Indo-Pacific origin but not from the Persian Gulf nor from other introduced populations in the eastern or central Mediterranean, therefore the precise origin remains to be discovered. Despite the small number of samples from the Persian Gulf compared to the Mediterranean, the haplotype diversity is higher in the native populations compared to the non-native.

Keywords: Non-indigenous species; Non-native; Balearic Islands; Species delimitation; Genetic population structure.

Introduction

The introduction of alien or non-indigenous species (NIS) to new geographic areas is considered one of the greatest threats to biodiversity and ecosystem structure and functioning, as NIS can compete with local species for resources, predate on them, or create novel habitats through ecosystem engineering (Ruiz *et al*., 1997; Tsirintanis *et al*., 2022). Despite not all being invasive, more than 1000 NIS (759 of them established) had been reported by December 2020 in the Mediterranean Sea, which is considered one of the marine regions with the most introduced species (Zenetos *et al*., 2022 a, b, c; Galanidi *et al*., 2023). The Suez Canal, opened in 1869, has become the main path for the entry of these species, which are commonly referred to as Erythrean aliens or Lessepsian migrants (e.g., Galanidi *et al*., 2023). Molluscs include the highest number of Mediterranean NIS with 230 species, 173 of them considered established (Zenetos *et al*., 2022 a).

Two NIS bivalve molluscs of the genus *Pinctada* Röding, 1798 have been reported in the Mediterranean: *Pinctada margaritifera* (Linnaeus, 1758) and *P. radiata* (Leach, 1814) (Zenetos *et al*., 2004). The black-lipped pearl oyster *Pinctada margaritifera* was imported around 1860 from the Red Sea for aquaculture but there is no evidence that it reached natural open sea environments (Zenetos *et al*., 2004). The rayed pearl oyster *Pinctada radiata* reported from the Mediterranean for the first time in 1874 in Alexandria (Monterosato, 1878), was probably the first Lessepsian migrant bivalve recorded (Gofas & Zenetos, 2003).

The taxonomic status of the morphologically similar species *P. radiata, Pinctada fucata* (A. Gould, 1850) and *Pinctada imbricata* Röding, 1798 (Scuderi *et al*., 2019) is unclear and some authors recognise them as subspecies of *P. imbricata* (Tëmkin, 2010; Barbieri *et al*., 2016; Gavrilović *et al*., 2017). This species complex is morphologically homogenous and shares the smaller sized shells and presence of hinge teeth (Morphogroup 2 according to Jameson, 1901). In contrast, *Pinctada mazatlanica* (Hanley, 1856), *Pinctada maxima* (Jameson, 1901) and *P. margaritifera* are larger species and lack the characteristic hinge teeth (Morphogroup 1 according to Jameson, 1901). These groups have also been assessed by molecular analyses (Tëmkin, 2010; Cunha *et al*., 2011).

The natural distribution range of *P. radiata* includes coastal habitats of the northern and western Indian Ocean, together with the Red Sea and Persian Gulf, whereas *P. imbricata* and *P. fucata* have been associated to tropical and subtropical littoral environments in the Western Atlantic and Indo-Pacific, respectively (Röding, 1798; Abbott, 1974; Chu, 2006; Wada & Tëmkin, 2008; Cunha *et al*., 2011; MolluscaBase, 2023a). Since the first record in the Mediterranean in the 19th century, *P. radiata* has been spreading to the west over the years and it is considered as well established (Scuderi *et al*., 2019). In the Balearic Islands, the species was first recorded in 1999, where a single individual was collected among fishing debris at 200 m of depth (Pons-Moyà & Pons, 2001). However, these records belong to a small specimen which could have easily been misidentified with a juvenile of *Pteria* sp. (Serge Gofas, pers. comm.). Since 2010 it has been reported in different localities of Mallorca and Menorca and is now considered well established (Ballesteros *et al*., 2020; Png-Gonzalez *et al*., 2021).

The genetic characterization of the non-indigenous and the native populations of *P. radiata* could clarify the taxonomic status of the species and infer the possible source and expansion pathways of introduction, which might help manage the species and minimise future introductions (Rius *et al*., 2015). For this purpose, the cytochrome c oxidase subunit 1 gene (COI) has served as a molecular marker to carry out many genetic studies in molluscs including *Pinctada* species (e.g., Giribet & Wheeler, 2002; Cunha *et al*., 2011; Meyer *et al*., 2013; Barbieri *et al*., 2013, 2016; Combosch *et al*., 2017; Gavrilović *et al*., 2017). Nonetheless, while *P. radiata* populations have already been studied throughout the eastern Mediterranean basin (Barbieri *et al*., 2013, 2016; Gavrilović *et al*., 2017), no genetic information is available from the western coasts including the Balearic Sea.Therefore, the main aims of this study are: 1) to assess the identity of the numerous specimens of *Pinctada,* found in the Balearic Islands, by molecular means; 2) to resolve the taxonomic status of the *P. imbricata* species group and resolve the species or subspecies ranks, and 3) to provide information on the genetic diversity of these populations in the Balearic Sea and compare with published data.

Materials and Methods

Sampling

Twenty-seven individuals were obtained along the Bay of Palma (Png-Gonzalez *et al*., 2021), two others by free diving in SW Mallorca and another two in Menorca (Ballesteros *et al*., 2020). Specimens were deposited at the University of the Balearic Islands collection (Table 1).

DNA Sequences generation and acquisition

About 1 mm^3 of adductor muscle or mantle was removed from each individual and placed in 50 µL of QuickExtract™ DNA Extraction Solution (Lucigen, Epicentre), heated at 65ºC for 3 hours and then at 95ºC for 5 minutes. Partial cytochrome oxidase subunit 1 (COI) mitochondrial gene was amplified with the universal primers LCO1490 (5′ GGT CAA CAA ATC ATA AAG ATA TTG G 3′) and HCO2198 (5′ TAA ACT TCA GGG TGACCA AAA AAT CA 3′) (Folmer *et al*., 1994). Polymerase chain reactions (PCR) were carried out in a final volume of 15 µL, containing 7.5 µL MyTaq™ Red DNA polymerase (Bioline), 0.6 µL of each primer (10 ng·µL^{-1}) and 50-80 ng of DNA. Amplification protocol started with an initial denaturing step at 95ºC for 4 min, followed by 35 cycles of denaturation at 95ºC for 40 s, annealing at 48ºC for 40 s and elongation at 72ºC for 1 min, and a final extending step at 72ºC for 6 min. PCR products, were checked with 1% agarose gel electrophoresis and purified using ExoSAP-IT™ Express PCR Product Cleanup (Thermofisher) Kit following manufacturer's protocol. Amplicons were sent to Eurofins (Germany) and Macrogen (Spain) for Sanger sequencing.

In addition, 30 available COI sequences of other *Pinctada* species were downloaded from GenBank (NCBI). Sequences of *Pteria* species (*Pteria sterna* (Gould, 1851), *P. hirundo* (Linnaeus, 1758) and *P. loveni* (Dunker, 1879), currently considered as a synonym of *Pteria gregata* (Reeve, 1857) in MolluscaBase 2023b) were used as the outgroup (Cunha *et al*., 2011) (Table 1, Table S1).

Genetic analyses

Consensus sequences of forward and reverse strands were generated, quality checked, and primer sequences eliminated with Geneious Prime 2020.2 (https://www.geneious.com). Consensus sequences were aligned with MAFFT 7.0 (G-INS-i iterative refinement method and 1PAM/κ2 parameter, Katoh *et al*., 2019). Flanking positions were trimmed to ensure information in at least 50% of positions.

Preliminary phylogenetic and species delimitation analyses considered a large dataset (n=63) with the sequences generated from present study and representatives of *Pteria sterna, Pteria hirundo, Pteria loveni,* (as the outgroup) *P. imbricata, P. fucata, P. radiata, P. margaritifera, P. mazatlanica, P. maxima,* and *P. martensii* (Dunker, 1880), the later currently considered a synonym of *P. fucata,* (MolluscaBase 2023c) (Table S1). Further analyses (phylogenetic and species delimitation) were performed with a smaller dataset (n=54) only including the target taxa (*P. radiata* and *P. imbricata*) and one representative for each *P. margaritifera*, *P. mazatlanica* and *P. maxima* in addition to the outgroup (*Pteria* spp.) (Table 1).

The best fitting nucleotide substitution model (HKY+F+G4 for both datasets) was determined with IQTREE (http://www.iqtree.cibiv.univie.ac.at) using the Bayesian Information Criterion (BIC). Best tree (-pers 0.2 and -numstop 4000) and support with 1000 fast bootstrap replicates (BS) were estimated under Maximum Likelihood (ML) criterion in IQTREE (Minh *et al*., 2013). Tree edition was made using FigTREE 1.4.4 (Rambaut, 2006) and Inkscape (Harrington *et al*., 2004).

Species delimitation analyses included tree-based

Table 1. Information of COI sequences used for the molecular analysis. Species name, locality (for those collected in the Balearic Islands; two specimens from Menorca identified between brackets) or region (for those downloaded from GenBank), geographic coordinates, individual identification code and GenBank accession number are given. Marinas are marked with an asterisk over the locality.

Species	Locality/Region	Coordinates	Identification code	GenBank
Pinctada radiata[1]	Cala Nova	39.5501º N, 2.5991º E	JA1os	OP056055
Pinctada radiata[1]	Can Pastilla*	39.5337º N, 2.7133º E	os1	OP056056
Pinctada radiata[1]	Cala Gamba*	39.5464º N, 2.6952º E	os2	OP056059
Pinctada radiata[1]	Cala Gamba*	39.5464º N, 2.6952º E	os6	OP056053
Pinctada radiata[1]	Portixol	39.5596º N, 2.6675° E	JA6os	OP056051
Pinctada radiata[1]	Portixol	39.5592° N, 2.6697° E	JA5os	OP056046
Pinctada radiata[1]	Portixol	39.5604° N, 2.6687º E	JA4os	OP056045
Pinctada radiata[1]	Portixol	39.5592° N, 2.6697º E	8pi	OP056039
Pinctada radiata[1]	Cala Nova*	39.5496º N, 2.5995º E	JA2os	OP056050
Pinctada radiata[1]	Cala Nova*	39.5496º N, 2.5995º E	2pi	OP056038
Pinctada radiata[1]	Arenal*	39.5024º N, 2.7471º E	os9	OP056054
Pinctada radiata[1]	S'Arenal	39.5020º N, 2.7491º E	pir1	OP056044
Pinctada radiata[1]	S'Arenal	39.5020º N, 2.7491º E	pir2	OP056049
Pinctada radiata[1]	S'Arenal	39.5020º N, 2.7491º E	pir3	OP056052
Pinctada radiata[1]	S'Arenal	39.5020º N, 2.7491º E	pir4	OP056042
Pinctada radiata[1]	S'Arenal	39.5020º N, 2.7491º E	pir5	OP056041
Pinctada radiata[1]	S'Arenal	39.5020º N, 2.7491º E	pir6	OP056047
Pinctada radiata[1]	S'Arenal	39.5020º N, 2.7491º E	pir7	OP056067
Pinctada radiata[1]	S'Arenal	39.5020º N, 2.7491º E	pir8	OP056048
Pinctada radiata[1]	S'Arenal	39.5020º N, 2.7491º E	pir9	OP056058
Pinctada radiata[1]	S'Arenal	39.5020º N, 2.7491º E	pir11	OP056043
Pinctada radiata[8]	Portixol	39.5591° N, 2.6696° E	E10	OP056040
Pinctada radiata[8]	Portixol	39.5596° N, 2.6675° E	E11	OP056057
Pinctada radiata[8]	Portixol	39.5501° N, 2.6697° E	21-4	OP056062
Pinctada radiata[8]	Portixol	39.5501° N, 2.6697° E	21-5	OP056064
Pinctada radiata[8]	Portixol	39.5501° N, 2.6697° E	21-6	OP056065
Pinctada radiata[8]	Portixol	39.5501° N, 2.6697° E	21-7	OP056066
Pinctada radiata[8]	Caló d'en Pellicer	39.5126° N, 2.4719° E	21-2	OP056063
Pinctada radiata[8]	Caló d'en Pellicer	39.5126° N, 2.4719° E	21-3	OP056068
Pinctada radiata[2]	Cala St. Antoni (Menorca)	39.8913° N, 4.2837° E	M3	OP056061
Pinctada radiata[2]	Riu pla (Menorca)	39.8934° N, 4.2733° E	M4	OP056060
Pinctada radiata[3]	Persian Gulf	-	-	KF284059.1
Pinctada radiata[3]	Persian Gulf	-	-	KF284060.1
Pinctada radiata[3]	Persian Gulf	-	-	KF284061.1
Pinctada radiata[3]	Persian Gulf	-	-	KF284062.1
Pinctada radiata[5]	Persian Gulf	-	-	GQ355875.1
Pinctada radiata[5]	Persian Gulf	-	-	GQ355876.1

Continued

Table 1 continued

Species	Locality/Region	Coordinates	Identification code	GenBank
Pinctada radiata[5]	Persian Gulf	-	-	GQ355877.1
Pinctada radiata[5]	Persian Gulf	-	-	GQ355878.1
Pinctada radiata[4]	Eastern Mediterranean	-	-	KT768194.1
Pinctada radiata[4]	Eastern Mediterranean	-	-	KT768196.1
Pinctada radiata[4]	Central Mediterranean	-	-	KT768197.1
Pinctada radiata[4]	Central Mediterranean	-	-	KT768198.1
Pinctada radiata[4]	Eastern Mediterranean	-	-	KT768199.1
Pinctada imbricata[5]	NW Atlantic	-	-	GQ355883.1
Pinctada imbricata[5]	NW Atlantic	-	-	GQ355873.1
Pinctada imbricata[5]	NW Atlantic	-	-	GQ355870.1
Pinctada imbricata[6]	NW Atlantic	-	-	KX713492.1
Pinctada margaritifera[5]	SW Indian	-	-	GQ355869.1
Pinctada maxima[5]	SW Pacific	-	-	GQ355881.1
Pinctada mazatlanica[5]	NE Pacific	-	-	AF374307.1
Pteria sterna[5]	-	-	-	GQ355874.1
Pteria hirundo[7]	-	-	-	AF120647.1
Pteria loveni[5]	NW Pacific	-	-	AB076925.1

[1]Png-Gonzalez *et al.,* 2021; [2]Ballesteros *et al*., 2020; [3]Meyer *et al*., 2013; [4]Barbieri *et al*., 2016; [5]Cunha *et al*., 2011; [6]Combosch *et al*., 2017; [7]Giribet & Wheeler 2002; [8]Present study.

analyses and genetic pairwise distances. The single and multi-rate Poisson Tree Processes model (PTP and mPTP, Zhang *et al*., 2013 and Kapli *et al*., 2017, respectively) was applied to the resulting ML tree to detect independently evolving entities (i.e., putative species). Both PTP and mPTP were run without the three outgroups and PTP with p-value of 0.01 and 0.05. The number of base substitutions per site from averaging over all sequence pairs within and between groups were calculated in MEGA X (Kumar *et al.,* 2018) using both *p*-distance and best fitting substitution model (Tamura-Nei in absence of HKY obtained in IQTREE, Tamura & Nei, 1993) and modelled with a gamma distribution (shape parameter = 4).

Haplotype diversity was calculated in *P. radiata* populations in Arlequin 3.5 (Excoffier & Lischer, 2010) (Persian Gulf, eastern Mediterranean, central Mediterranean, Mallorca and Menorca). All ambiguous positions were removed for each sequence pair. F_{ST} genetic distances were calculated in Arlequin 3.5 to compare the genetic variance between populations. In addition, a hierarchical analysis of molecular variance (AMOVA) was calculated based on 10000 random iterations using Arlequin 3.5, to compare sequences between and within populations (Mallorca, Menorca, central Mediterranean, eastern Mediterranean and Persian Gulf) and between regions (western Mediterranean, central + eastern Mediterranean and Persian Gulf).

The genetic structure of populations of *P. radiata* was examined by means of parsimony haplotype network in TCS v1.23 (Clement *et al*., 2000), assuming IUPAC ambiguity codes as missing data. Sequences from Barbieri *et al*. (2016), are shorter (385 bp) than those generated for the present study and others in GenBank at the beginning of the alignment (600 bp). On the contrary, some sequences from this study, although long (>513 bp), have missing data at the end of the alignment. The haplotype network analyses of all sequences mask all sites with missing data which, in this case, would leave only 299 bp and, thus, disregards sites that actually hold genetic variation outside this region. A different approach would be to exclude shorter sequences from analyses. In this study, three separate sets of analyses were run with PopART 1.7 (Leigh & Bryant, 2015) (Fig. S1): a first one, without the <560 bp-long sequences obtained in this study, because those shorter sequences masked sites with missing information in the 3’ end of the alignment; a second one, removing, in addition, those from Barbieri *et al*. (2016) that masked sites with missing information in the 5’ end of the alignment (Fig. S1); and the third network excluding all the others and in addition those from the Persian Gulf from Cunha *et al*. (2011) (Fig. S1). The results obtained from these analyses were combined by hand with Inkscape (Harrington *et al*., 2004).

Results

The 31 *P. radiata* partial COI amplicons generated in this study were 513-680 base pairs long (GenBank accession numbers OP056038 to OP056068; Table 1), becoming 440-600 bp of length after removing primer sequences and trimming bad quality ends.

Balearic individuals belong to six haplotypes: H1 is the most prevalent, with 26 sequences collected from different sites in Mallorca and Menorca and the other haplotypes (H2-H6) are represented by single sequences all collected in the Bay of Palma, and only differing from H1 in one or two mutations (Fig. 1B). Neither of these haplotypes have been previously published. When compared with other regions, haplotype diversity is found higher amongst the individuals collected from the Persian Gulf (0.786) than within the non-native Mediterranean (0.605). Comparing only the Mediterranean populations the higher haplotype diversity values is eastern Mediterranean region (0.403), followed by Mallorca (0.320) and central Mediterranean (0.198) (Table 2).

Alignment of the large dataset (63 sequences, Fig. S2, Table S1) included 338 parsimony-informative sites of the 600 analysed, whereas the smaller dataset (51 sequences of the same length, Fig. 1C, Table 1) included 221. Both phylogenetic analyses recovered *P. radiata* as a well-supported clade (Fig. 1C, BS = 94; Fig. S2, BS = 83). Sequences of *P. radiata* were clustered according to their geography, with the Balearic Islands clade (orange

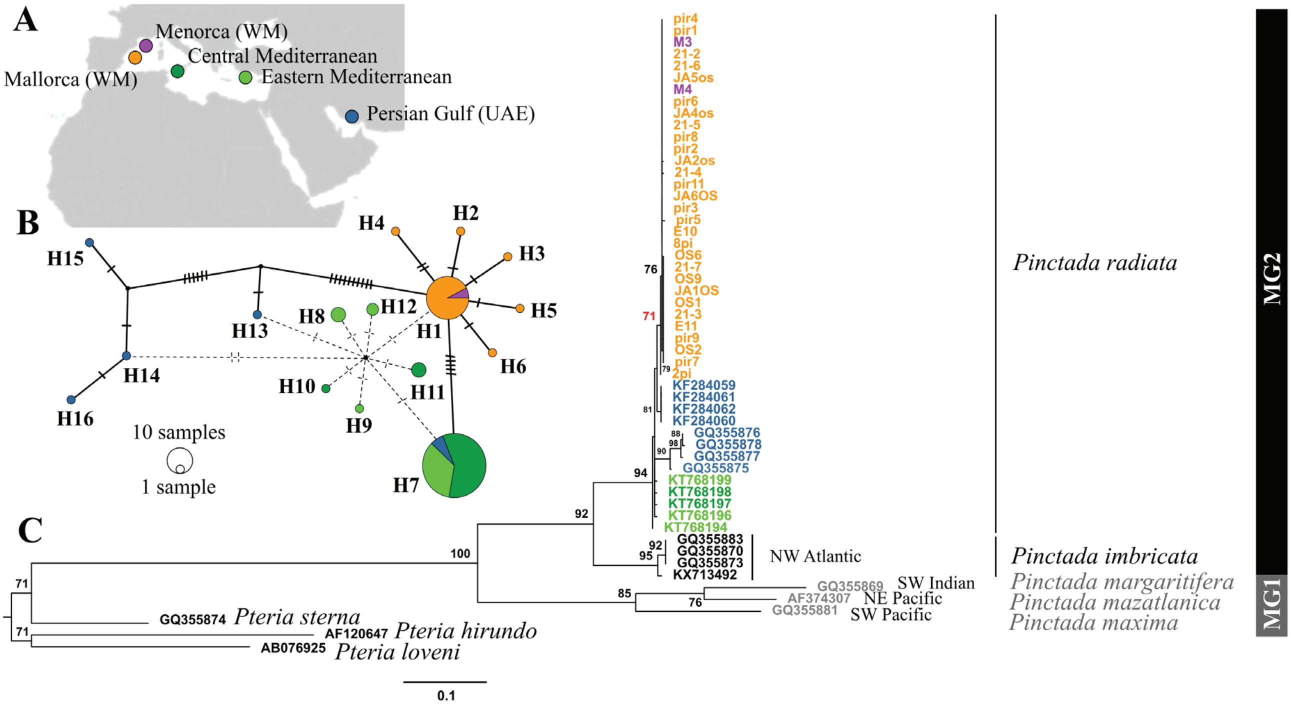

Fig. 1: A) Geographic location and colour legend of the *P. radiata* populations included in the genetic analysis. B) COI haplotype network with (dashed line) and without (solid line) short sequences (Barbieri *et al*., 2016); each haplotype is represented by one circle, whose size is directly proportional to the frequency of the haplotype; every hatch mark represents a nucleotide mutation between haplotypes and hypothetical or non-sampled haplotypes are represented by black nodes. C) Maximum-likelihood topology of COI sequences considering *Pteria* species; MG1 and MG2 symbolise the two different morphogroups described by Jameson (1901); maximum-likelihood bootstrap support values are represented by numbers near the nodes (that of the Balearic population in red); the bar indicates the degree of evolutionary divergence.

Table 2. Geographic distribution of *Pinctada radiata* haplotypes used for the analysis. Total number of individuals per population (N), number of haplotypes (H) and corresponding codes (specific number of individuals per haplotype between brackets and singletons indicated in bold) and haplotype diversity (H) are shown. Abbreviations: CEM, central-eastern Mediterranean; CM, central Mediterranean; EM, eastern Mediterranean; ML, Mallorca; MN, Menorca; PG, Persian Gulf; WM, western Mediterranean.

Region	Population	N	H	H codes	H Diversity
WM (current study)	ML	29	6	H1 (24), **H2, H3, H4, H5, H6**	0.320 ± 0.112
	MN	2	1	H1 (2)	0.000 ± 0.000
CEM	CM	38	3	H7 (34), **H10**, H11 (3)	0.198 ± 0.083
	EM	26	4	H7 (20), H8 (3), **H9**, H12 (2)	0.403 ± 0.113
PG	PG	8	5	H7 (4), **H13, H14, H15, H16**	0.786 ± 0.151

and purple terminals in Fig. 1C, BS = 71; Fig. S2) sister and only slightly divergent to the Persian Gulf clade (blue terminals in Fig. 1C, BS = 81; Fig. S2). This result provides evidence of the presence of the invasive oyster *P. radiata* in the Balearic Archipelago. Specimens from GenBank identified as *P. imbricata* were also recovered as a well-supported clade (black terminals in Fig. 1C, BS = 95; Fig. S2, BS = 91) sister to *P. radiata* (BS = 92). The single rate Poisson tree processes (PTP) method with p-value 0.01 and 0.05 performed with the smaller dataset recovered *P. maxima*, *P. mazatlanica*, *P. margaritifera*, *P. imbricata* and *P. radiata* as putative species, while the multiple rate Poisson tree processes (mPTP) lumped the three singletons identified as *P. maxima, P. mazatlanica* and *P. margaritifera* in the same cluster.

The haplotype network analysis without shorter sequences recovered higher genetic distances and number of haplotypes than the network including all sequences due to the high number of variable sites that are masked in those positions with missing information. The meta-haplotype network made after combining the long and short alignments together with genetic distance analysis revealed a strong geographic structure within the dataset, with low genetic distance among Balearic individuals (~0.05 %) and higher genetic divergence to those from the central and eastern Mediterranean (~0.60 %) and from the Persian Gulf (~2.20 %, Table S1, Fig. 1B). The distances amongst geographical regions are considerably reduced when the short sequences are added to the haplotype network (dotted lines in Fig. 1B; Fig. S1).

Individuals from Mallorca and Menorca were regarded as the same population for the Pairwise F_{ST} analyses, since the two sequences from Menorca belong to the most prevalent Mallorcan haplotype. Significant differences were found between the four considered populations (Persian Gulf, eastern Mediterranean, central Mediterranean and Mallorca + Menorca). However, genetic distances between Mallorca + Menorca, and the other three populations are the highest (Table S3). In contrast, the Pairwise distances between the native populations from the Persian Gulf and those from central and eastern Mediterranean are low because they all share the H7 haplotype.

Analysis of molecular variance (AMOVA) among all regions revealed high genetic variation among regions (78.94%) and low genetic differentiation among populations (0.00%) and within populations (21.49 %). These results indicate there are low levels of gene flow between regions (Table S4). The comparison of only the Mediterranean regions highlights the difference of the western Mediterranean populations where the variability between regions is 89.10% (Table S4).

Discussion

Pinctada radiata is one of the NIS recently established and rapidly spreading in the benthic environments of the Balearic Islands. However, the populations found in the archipelago are still not reaching densities of >4 individuals/100 m^2 (Ballesteros *et al*., 2020; Png-Gonzalez *et al*., 2021; personal observations) which is still far less than the dense aggregations of up to 145 individuals/m^2 recorded from *Posidonia oceanica* meadows in Turkey (Derbali *et al*., 2011). Most specimens collected in the Balearic Islands were found in shallow hard bottom environments (less than 2 m deep), and were especially abundant in environments exposed to high anthropogenic activities, such as harbors and marinas.

Results of the phylogenetic analyses provide evidence of the monophyly of *P. radiata* including the Balearic populations, confirming the identification, and the close relationship of this taxon to *P. imbricata*, as previously shown in other molecular phylogenies of the group (Tëmkin, 2010; Cunha *et al*., 2011). Species delimitation analyses, including genetic distances, statistical parsimony haplotype network and tree-based analyses, showed *P. radiata* deserves the species rank and thus the use of the subspecies name should be discouraged.

The available sequences of the Mediterranean *P. radiata* belong to 12 haplotypes some of which have not been found amongst indigenous populations. Therefore, although the potential geographic area of origin cannot be assumed due to the absence of published haplotypes, it is likely that the haplotypes found in the Balearic Islands came from natural populations, either in a single event of many genetically diverse individuals or in multiple events translocating specimens from one or several different populations, indicating the maritime transport as the main vector (as previously reported in Png-Gonzalez *et al*., 2021). The lack of comprehensive genetic surveys in the species native distribution range and the short length of the sequences published in some previous studies (385 bp in Barbieri *et al*., 2016) may be responsible for such results.

Nevertheless, and beside the small number of samples from the Persian Gulf compared to the Mediterranean (8 and 95 sequences, respectively), the haplotype diversity is higher in the native populations (0.786) compared to the non-native (0.605). The genetic variability of alien species is expected to be lower out of their native range (Allendorf & Lundquist, 2003), although there are some examples in the literature where the genetic diversity of species outside their native range is higher due to rapid evolution after invasion or multiple invasion events (e. g. Lee, 2002; Hassan *et al*., 2003; Parker *et al*., 2003; Azzurro *et al*., 2006). Some Lessepsian invaders have, in fact, been reported to show a similar or even a higher genetic diversity in Mediterranean populations compared to those in their native habitats (e. g., Hassan *et al*., 2003; Bariche & Bernardi, 2009; Barbieri *et al*., 2011; Bernardi *et al*., 2016; Azzurro *et al*., 2022).

The present study constitutes the first genetic survey of *P. radiata* in the western Mediterranean and a step forward in assessing the correct identification and genetic structure of the species in its non-native range. Nevertheless, the current available molecular data hinders the identification of its introduction pathway in the Balearic Islands and highlights the need for more genetic studies in a broader range of native populations.

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CRediT authorship contribution statement: Joseba Aguilo-Arce: Conceptualization, Methodology, Formal analysis, Data curation, Writing – original draft, Writing – review & editing, Visualization, Validation. Joana F. Ferragut: Formal analysis, Writing – review & editing. Lydia Png-Gonzalez: Methodology, Writing – review & editing. Aina Carbonell: Conceptualization, Writing – review & editing. María Capa: Conceptualization, Methodology, Writing – original draft, Formal analysis, Writing – review & editing, Funding acquisition.

Declaration of competing interest: The authors declare no competing interests.

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APPENDIX

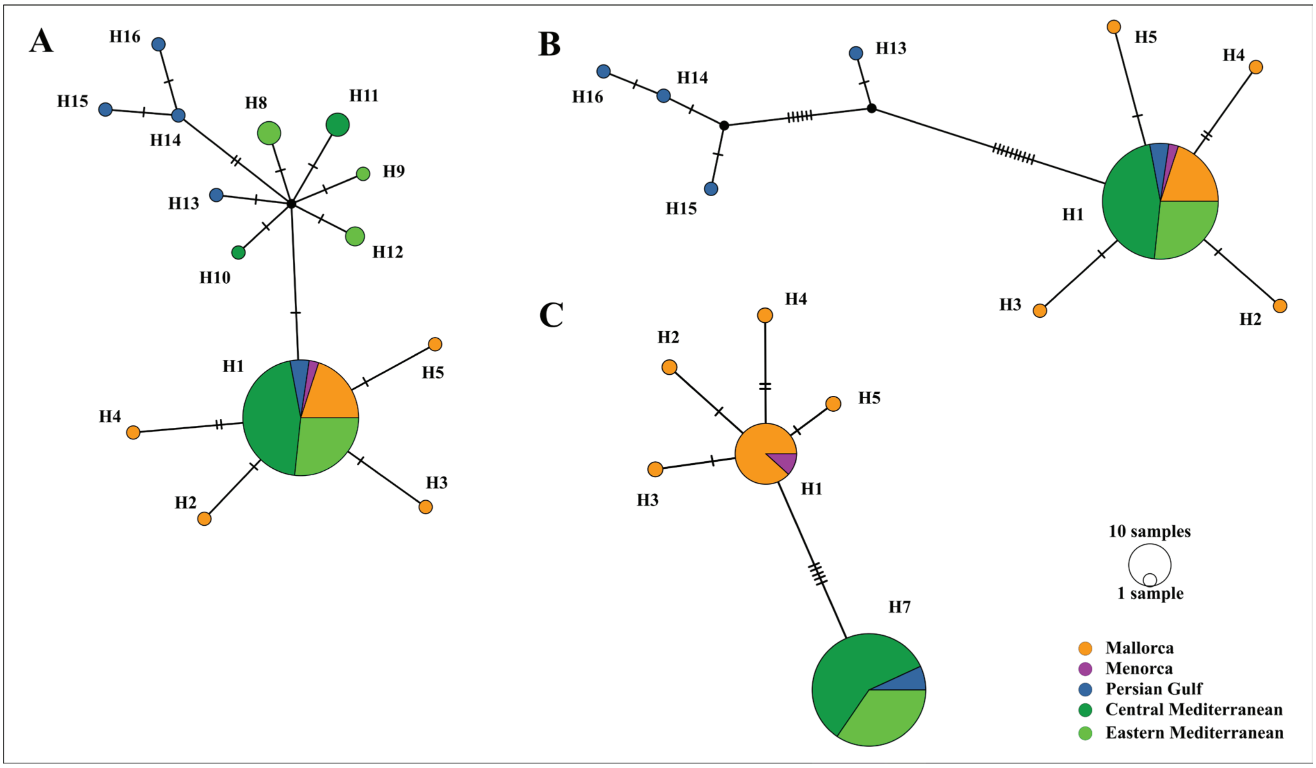

Fig. S1: COI haplotype networks used to create the meta-haplotype network showed in Fig. 1. A) network without <560 bp-long sequences from this study (therefore sequence H6 was excluded); B) network without previous sequences and also without those from Barbieri *et al*. (2016); C) network without previous and also without those from Cunha *et al*. (2011) from the Persian Gulf. Each haplotype is represented by one circle, whose size is directly proportional to the frequency of the haplotype; every hatch mark represents a nucleotide mutation between haplotypes and hypothetical or non-sampled haplotypes are represented by black nodes.

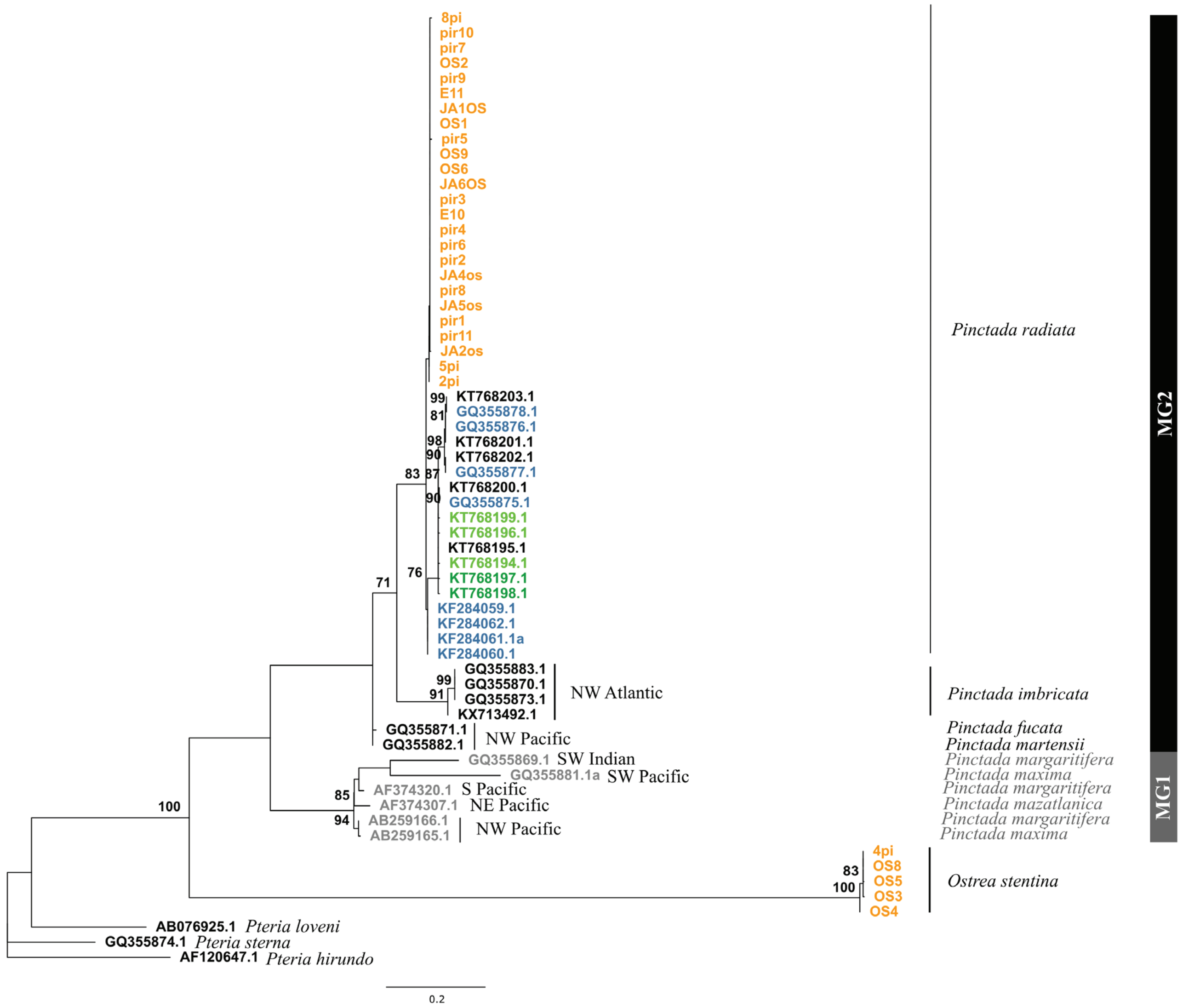

Fig. S2: Maximum-likelihood topology of the large COI sequence dataset considering *Pteria* spp.; *P. radiata* sequence origin is represented by colours: orange (Mallorca), blue (Persian Gulf), light (eastern Mediterranean) and dark green (central Mediterranean). MG1 and MG2 symbolise the two different morphogroups described by Jameson (1901); maximum-likelihood bootstrap support values are represented by numbers near the nodes (that of the Balearic population in red); the bar indicates the degree of evolutionary divergence.

Table S1. Information of COI sequences used for the molecular analysis of the larger dataset. Species name, locality (for those collected in the Balearic Islands) or region (for those downloaded from GenBank), geographic coordinates, individual identification code and GenBank accession number are given. Marinas are marked with an asterisk over the locality. The added sequences comparing with the smaller dataset are indicated in bold.

Species	Locality/Region	Coordinates	Identification code	GenBank
Pinctada radiata[1]	Cala Nova	39.5501º N, 2.5991º E	JA1os	OP056055
Pinctada radiata[1]	Can Pastilla*	39.5337º N, 2.7133º E	os1	OP056056
Pinctada radiata[1]	Cala Gamba*	39.5464º N, 2.6952º E	os2	OP056059
Pinctada radiata[1]	Cala Gamba*	39.5464º N, 2.6952º E	os6	OP056053
Pinctada radiata[1]	Portixol	39.5596º N, 2.6675° E	JA6os	OP056051
Pinctada radiata[1]	Portixol	39.5592° N, 2.6697° E	JA5os	OP056046
Pinctada radiata[1]	Portixol	39.5604° N, 2.6687º E	JA4os	OP056045
Pinctada radiata[1]	Portixol	39.5592° N, 2.6697º E	8pi	OP056039
Pinctada radiata[1]	Cala Nova*	39.5496º N, 2.5995º E	JA2os	OP056050
***Pinctada radiata*[1]**	**Cala Nova***	**39.5496º N, 2.5995º E**	**5pi**	**-**
Pinctada radiata[1]	Cala Nova*	39.5496º N, 2.5995º E	2pi	OP056038
Pinctada radiata[1]	Arenal*	39.5024º N, 2.7471º E	os9	OP056054
Pinctada radiata[1]	S'Arenal	39.5020º N, 2.7491º E	pir1	OP056044
Pinctada radiata[1]	S'Arenal	39.5020º N, 2.7491º E	pir2	OP056049
Pinctada radiata[1]	S'Arenal	39.5020º N, 2.7491º E	pir3	OP056052
Pinctada radiata[1]	S'Arenal	39.5020º N, 2.7491º E	pir4	OP056042
Pinctada radiata[1]	S'Arenal	39.5020º N, 2.7491º E	pir5	OP056041
Pinctada radiata[1]	S'Arenal	39.5020º N, 2.7491º E	pir6	OP056047
Pinctada radiata[1]	S'Arenal	39.5020º N, 2.7491º E	pir7	OP056067
Pinctada radiata[1]	S'Arenal	39.5020º N, 2.7491º E	pir8	OP056048
Pinctada radiata[1]	S'Arenal	39.5020º N, 2.7491º E	pir9	OP056058
***Pinctada radiata*[1]**	**S'Arenal**	**39.5020º N, 2.7491º E**	**pir10**	**-**
Pinctada radiata[1]	S'Arenal	39.5020º N, 2.7491º E	pir11	OP056043
Pinctada radiata[8]	Portixol	39.5591° N, 2.6696° E	E10	OP056040
Pinctada radiata[8]	Portixol	39.5591° N, 2.6696° E	E11	OP056057
Pinctada radiata[3]	Persian Gulf	-	-	KF284059.1
Pinctada radiata[3]	Persian Gulf	-	-	KF284060.1
Pinctada radiata[3]	Persian Gulf	-	-	KF284061.1
Pinctada radiata[3]	Persian Gulf	-	-	KF284062.1
Pinctada radiata[5]	Persian Gulf	-	-	GQ355875.1
Pinctada radiata[5]	Persian Gulf	-	-	GQ355876.1
Pinctada radiata[5]	Persian Gulf	-	-	GQ355877.1
Pinctada radiata[5]	Persian Gulf	-	-	GQ355878.1
Pinctada radiata[4]	Eastern Mediterranean	-	-	KT768194.1
***Pinctada radiata*[4]**	**-**	**-**	**-**	**KT768195.1**
Pinctada radiata[4]	Eastern Mediterranean	-	-	KT768196.1
Pinctada radiata[4]	Central Mediterranean	-	-	KT768197.1

Continued

Table S1 continued

Species	Locality/Region	Coordinates	Identification code	GenBank
Pinctada radiata[4]	Central Mediterranean	-	-	KT768198.1
Pinctada radiata[4]	Eastern Mediterranean	-	-	KT768199.1
***Pinctada radiata*[4]**	**-**	**-**	**-**	**KT768200.1**
***Pinctada radiata*[4]**	**-**	**-**	**-**	**KT768201.1**
***Pinctada radiata*[4]**	**-**	**-**	**-**	**KT768202.1**
***Pinctada radiata*[4]**	**-**	**-**	**-**	**KT768203.1**
Pinctada imbricata[5]	NW Atlantic	-	-	GQ355883.1
Pinctada imbricata[5]	NW Atlantic	-	-	GQ355873.1
Pinctada imbricata[5]	NW Atlantic	-	-	GQ355870.1
Pinctada imbricata[6]	NW Atlantic	-	-	KX713492.1
***Pinctada fucata*[5]**	**NW Pacific**	**-**	**-**	**GQ355871.1**
***Pinctada martensii*[5]**	**NW Pacific**	**-**	**-**	**GQ355882.1**
Pinctada margaritifera[5]	SW Indian	-	-	GQ355869.1
***Pinctada margaritifera*[5]**	**NW Pacific**	**-**	**-**	**AB259166.1**
***Pinctada margaritifera*[5]**	**S Pacific**	**-**	**-**	**AF374320.1**
Pinctada maxima[5]	SW Pacific	-	-	GQ355881.1
***Pinctada maxima*[5]**	**NW Pacific**	**-**	**-**	**AB259165.1**
Pinctada mazatlanica[5]	NE Pacific	-	-	AF374307.1
***Ostrea stentina*[8]**	**Cala Gamba***	**39.5466º N, 2.6952º E**	**os3**	**-**
***Ostrea stentina*[8]**	**Cala Gamba***	**39.5466º N, 2.6952º E**	**os4**	**-**
***Ostrea stentina*[8]**	**Cala Gamba***	**39.5466º N, 2.6952º E**	**os5**	**-**
***Ostrea stentina*[8]**	**Cala Gamba***	**39.5466º N, 2.6952º E**	**os8**	**-**
***Ostrea stentina*[8]**	**Portixol**	**39.5592° N, 2.6697° E**	**4pi**	**-**
Pteria sterna[5]	-	-	-	GQ355874.1
Pteria hirundo[7]	-	-	-	AF120647.1
Pteria loveni[5]	NW Pacific	-	-	AB076925.1

[1]Png-Gonzalez *et al.,* 2021; [2]Ballesteros *et al*., 2020; [3]Meyer *et al*., 2013; [4]Barbieri *et al*., 2016; [5]Cunha *et al*., 2011; [6]Combosch *et al*., 2017; [7]Giribet & Wheeler 2002; [8]Present study.

Table S2. Intra and inter-population mean genetic distances between *Pinctada radiata* groups and the congeneric *Pinctada imbricata* based on mitochondrial COI marker. Within-group distance is highlighted in grey separating between-group distances calculated following *p*-distance method (lower left) or Tamura-Nei model (upper right). Abbreviations: CM, central Mediterranean; EM, eastern Mediterranean; ML, Mallorca; MN, Menorca; PG, Persian Gulf; Pi, *Pinctada imbricata*).

	ML+MN	**EM**	**CM**	**PG**	**Pi**
ML+MN	0.0005	0.0061	0.0061	0.0224	0.1616
EM	0.0060	0.0052	0.0052	0.0069	0.1497
CM	0.0060	0.0052	0.0052	0.0069	0.1532
PG	0.0219	0.0068	0.0068	0.0206	0.1629
Pi	0.1354	0.1253	0.1279	0.1368	0.0071

Table S3. Pairwise F_{ST} distances (lower left) and p-values (upper right) calculated considering all populations separately, using COI mitochondrial DNA marker. Significant p-values are labelled in bold. Scale grey colouring was used to indicate genetic distances, being white the minimum distance (0) and dark grey the maximum (1). Abbreviations: CM, central Mediterranean; EM, eastern Mediterranean; ML, Mallorca; MN, Menorca; PG, Persian Gulf.

	ML + MN	**CM**	**EM**	**PG**
ML + MN	-	**0.00000+-0.0000**	**0.00000+-0.0000**	**0.00000+-0.0000**
CM	0.92024	-	**0.02703+-0.0194**	**0.00000+-0.0000**
EM	0.87174	0.04301	-	**0.00000+-0.0000**
PG	0.72765	0.54882	0.36131	-

Table S4. Hierarchical analyses of molecular variance (AMOVA) computed among all regions (western Mediterranean, central + eastern Mediterranean, and Persian Gulf) and with only Mediterranean regions (western Mediterranean and central + eastern Mediterranean). Calculations were performed after a permutation test with 10000 iterations.

Source of variation	**df**	**Variance component**	**Percentage of variance**	**Fixation Indices**	**P-value**
Among regions	2	1.92866 (Va)	78.94	F_{CT} = 0.78936	0.06851
Among populations within regions	2	-0.01041 (Vb)	-0.43	F_{SC} = -0.02023	0.23218
Within populations	98	0.52508 (Vc)	21.49	F_{ST} = 0.78510	< 0.00001
Among Mediterranean regions	1	2.11692 (Va)	89.1	F_{CT} = 0.89102	0.33624
Among populations within Mediterranean regions	2	0.00527 (Vb)	0.22	F_{SC} = 0.02036	0.23812
Within populations	91	0.25365 (Vc)	10.68	F_{ST} = 0.89102	< 0.00001